AD _____

GRANT NUMBER: DAMD17-97-1-7013

TITLE: Cyclin D1, Anchorage-Independent Growth and Breast Cancer

PRINCIPAL INVESTIGATOR: Catherine Welsh, M.D.

CONTRACTING ORGANIZATION: University of Miami
Miami, Florida 33101

REPORT DATE: October 1998

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

The views, opinions and/or findings contained in this report are those of the author (s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1998	Annual (15 Sep 97-14 Sep 98)

4. TITLE AND SUBTITLE
Cyclin D1, Anchorage-Independent Growth and Breast Cancer

5. FUNDING NUMBERS
DAMD17-97-1-7013

6. AUTHOR(S)
Welsh, Catherine, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Miami
Miami, Florida 33101

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Derangements in the cell cycle machinery contribute to uncontrolled cell growth and tumorigenesis. Regulators of G1 progression including cyclin D1 and the cyclin-dependent kinase inhibitors p21 and p27 appear to be of particular importance in the pathogenesis of breast cancer. Our data show that the overexpression of cyclin D1 leads to a compensatory increase in the cdk inhibitor p21, and suggest that this increase can weaken the value of cyclin D1 overexpression as a diagnostic indicator. We suggest that aggressive breast cancer will involve both (i) overexpression of cyclin D1 and (ii) the failure to undergo a compensatory upregulation of cdk inhibitors. The specific aims are designed to test these hypotheses in cell culture models, nude mice, and breast cancer biopsies. We have thus far determined that p21-null fibroblasts are adhesion-dependent and suitable for use to study the role of p21 in negating the effect of cyclin D1 overexpression on anchorage-independent cell cycle progression. We have also stably transfected cells with a tetracycline-regulated cyclin D1 to further address this issue. Finally, we have optimized the conditions for the immunohistochemical analysis of human breast cancer tissue for cyclin D1 and p27 expression.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	18
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

__✓__ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

__✓__ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

____ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

____ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Catherine Fulbright 10/12/98

PI - Signature Date

TABLE OF CONTENTS

INTRODUCTION

An increasing body of evidence suggests that derangements in the cell cycle machinery contribute to uncontrolled cell growth and tumorigenesis. Proteins which regulate progression through the G1 phase, including cyclin D1 and the cyclin-dependent kinase inhibitors p21 and p27, appear to be of particular importance in the pathogenesis of breast cancer. Cyclin D1 gene amplification and/or mRNA overexpression occur in a significant portion of human breast cancers (1-5). Moreover, transgenic mice overexpressing cyclin D1 in mammary tissue develop breast hyperplasia and carcinomas, which are frequently multifocal (6). In addition, recent studies show that low p21 and p27 protein levels in breast cancer biopsies correlate significantly with tumor aggressiveness, histologic grade, and decreased overall patient survival (7-11).

As positive and negative regulators of proliferation, respectively, the levels of cyclins and cdk inhibitors relative to each other determine whether progression through G1 phase proceeds (12-16). The levels of cyclin D1 and p21 and p27 are controlled by growth factors and cell adhesion to the extracellular matrix (17,18). This regulation underlies the anchorage- and mitogen-dependence of G1 progression in normal cells. Conversely, anchorage- and mitogen-independent growth is the hallmark of tumorigenesis.

The consequences of cyclin D1 overexpression on breast tumor cell proliferation has yet to be established. Several cyclin D1-overexpressing cells have similar, or even somewhat delayed cell cycle kinetics, compared to normal mammary epithelial cells (4,19,20). Moreover, the overexpression of cyclin D1 fails to induce anchorage-independent growth, the best correlate of tumorigenicity (unpublished observations). Our preliminary data show that the consequences of cyclin D1 overexpression will be most apparent when examining anchorage-independent growth because that is the condition in which normal levels of cyclin D1 become rate-limiting. In addition, our data show that the overexpression of cyclin D1 leads to a compensatory increase in the cdk inhibitor p21, and that this compensatory increase can counteract the expected cyclin D1 effect on anchorage-independent Rb phosphorylation and cell cycle progression. Thus, we proposed that the value of cyclin D1 overexpression as a diagnostic indicator for breast cancer is weakened by the compensatory upregulation of cdk inhibitors (CKIs) that can occur in breast cancer cells. We further suggest that aggressive breast cancer will involve both (i) overexpression of cyclin D1 and (ii) the failure to undergo a compensatory upregulation of cdk inhibitors. The specific aims outlined below are designed to test these hypotheses in cell culture models, nude mice, and breast cancer biopsies. Aim 1 is to show that compensatory upregulation of CKIs negates the effect of cyclin D1 overexpression in inducing anchorage-independent growth. Aim 2 is to determine if compensatory upregulation of CKIs negates the effect of cyclin D1 overexpression on tumor formation. Aim 3 is to determine if the overexpression of cyclin D1 in breast cancer cell lines has a more pronounced growth effect if the cells have also lost their ability to upregulate CKIs. Aim 4 is to examine the relative expression of cyclin D1 and CKIs in a series of breast cancer biopsies.

BODY

Aim 1

Task 1. Complete characterization of adhesion-dependent phenotype of wild-type, p21, and p27 null mouse embryo fibroblasts (MEFs).

We have characterized the G1 cell cycle events with regard to the effects of cellular adhesion and compared p21-null, p27-null, and wild type mouse embryo fibroblasts (Fig 1). Cells were rendered quiescent by serum starvation and stimulated with mitogens, both in monolayer and suspension. Collected cells were lysed and analyzed by western blotting and in vitro kinase assays. The results showed that when cultured in monolayer, the p21-null cells have a shortened G1 phase. When studied in suspension (and consistent with the preliminary results in the application), we find that p21-null MEFs have partially lost their adhesion requirement for activation of cyclin E-cdk2, but retain normal adhesion requirements for expression of cyclin D1, phosphorylation of pRb and expression of cyclin A. Likewise, p27-null cells exhibited partial activation of cyclin E-cdk2 in G0. Since this partial cyclin E activity failed to result in subsequent Rb phosphorylation, these cells still exhibit an adhesion-dependent phenotype. These studies have now been repeated several times with similar results and we feel that the findings have been confirmed.

Task 2. Obtain tetracycline-cyclin D1 transfectants in mouse embryo fibroblasts. Analyze the tetracycline-cyclin D1 cells (wild-type and knock-out) for their ability to undergo anchorage-independent growth. Compare the rates and extent of Rb phosphorylation, cyclin D1 and E kinase activities and cyclin A expression when the transfectants are cultured in the presence and absence of substratum.

With regard to task 2, we have transfected wild-type MEFs with the tetracycline-regulated cyclin D1 expression vectors and obtained several clones. We observed that, in contrast to NIH-3T3 cells, MEFs are inefficient in their ability to form stable transfectants. We analyzed 30 clones by western blotting and immunofluorescence using anti-cyclin D1 antibodies, but have found only one that nicely expresses cyclin D1 in a tetracycline-regulated manner (Fig 2). We are currently determining whether this clone is stable in culture. We had also transfected p21 and p27 null MEFs with the tetracycline-regulated vectors. These transfections have not yielded the desired clones, and are complicated by the fact that the knock-out cells are G418 resistant prior to the transfection. Thus, we are not able to double select for expression of the two vectors needed to yield tetracycline-regulated cells. We do select with hygromycin, the second selectable marker in the tetracycline-regulated system, but the inability to use double drug selection probably explains our inability to obtain clones. We are presently considering whether to subclone cyclin D1 into a tet-regulated retrovirus in order to circumvent these problems with low efficiency transfection.

Aim 4

Task 1. Develop and quantify the immunohistochemical procedures for the analysis of breast cancer biopsies. Optimize the specific conditions for antibody-detection of protein expression in formalin-fixed paraffin-embedded tissue.

We have analyzed antibodies specific for cyclin D1 and p27 in formalin-fixed paraffin-embedded tissue. For each of these antibodies, we have determined the conditions for optimal staining while minimizing background signals. Tissue sections undergo antigen retrieval by exposure to steam heat at 95 degrees C for 20 minutes. The optimized pH for antigen retrieval is 9 for anti-p27 and 6 for anti-cyclin D1. Sections are incubated with the primary antibody for 3 hours at room temperature at a dilution of 1:10,000 for anti-p27 and 1:250 for anti-cyclin D1. Subsequent incubation with secondary antibody and development with DAB chromogen is used following standard procedures. Counterstaining is with fast green. Subsequent experiments will elucidate the optimal procedure for p21 antibodies.

Task 2. Obtain high- and low-expression controls for cyclin D1, p21, and p27 antibodies by preparing blocks from cell lines which over- or under-express the cognate protein.

We have prepared formalin-fixed paraffin-embedded cell blocks from NIH-3T3 cells. Cells that serve as high expressers of p27 are obtained by serum starvation of cell monolayers prior to collection. Low expressers are synchronized cells that have been stimulated to cycle in monolayer prior to collection. Using the optimized conditions determined in Task 1, all high p27 expression control cells exhibit strong nuclear staining (Fig 3) whereas low p27 expression controls exhibit low levels of expression (Fig 4). Negative control cells, in which the primary antibody is omitted, completely lack nuclear staining (Fig 5).

We have also prepared formalin-fixed paraffin-embedded cell blocks from NIH-3T3 cells which are stably transfected with a tetracycline-regulated cyclin D1 expression vector. Cells grown in the absence of tetracycline (expression of ectopic cyclin D1 is induced) serve as high cyclin D1 expression controls. Conversely, serum-starved cells grown in the presence of tetracycline (expression of ectopic cyclin D1 is repressed) serve as low expression controls for cyclin D1. All high expression control cells exhibit strong nuclear staining (Fig 6), whereas low expression controls lack any nuclear staining (Fig 7). Negative control cells, in which the primary antibody is omitted, completely lack nuclear staining (Fig 8). Additional blocks will be made for p21 controls using wild-type and p21-null fibroblasts.

Task 3. Identify human breast cancer tissue specimens which fulfill the requirements for good and poor prognostic factors. Prepare tissue sections of these specimens in a blinded fashion for evaluation.

We have begun to analyze and pull breast cancer tissue specimens that fulfill the characteristics of good and poor prognosis tumors. This will continue as we initiate the staining of such tissues using the methods outlined above.

The remaining tasks for aims1 and 4 as well as the tasks for aims 2 and 3 are scheduled to begin during the second year and continue into year 3.

CONCLUSIONS

1. p21-null and p27-null MEFs are anchorage-dependent and suitable for the proposed studies. They have a shortened G1 phase and have partially lost normal control of cyclin E-cdk2 activity. Both of these phenotypes are expected and consistent with the known inhibitory roles of p21 and p27.

2. Preliminary experiments indicate that we have obtained a tetracycline-regulated cyclin D1 in wild-type MEFs. Analysis of these cells for effects of cyclin D1 on p21 is underway.

3.Technical problems have prevented us from obtaining a tetracycline-regulated cyclin D1 in p21-null MEFs. Use of a cyclin D1/tet-regulated retrovirus (rather than plasmid) is now being considered to overcome this problem.

4. Antibodies specific for cyclin D1 and p27 can be used to detect protein expression in formalin-fixed paraffin-embedded tissue using the optimized protocol. Using cell blocks prepared for positive and negative controls as described, we will begin choosing a panel of breast cancer tissue specimens for analysis.

REFERENCES

1. Lammie GA, Fantl V, Smith R, Shuuring E, Brookes S, Michalides R, Dickson C, Arnold A, and Peters G (1991) D11S287, a putative oncogene on chromosome 11q13, is amplified and expressed in squamous cell and mammary carcinomas and linked to BCL-1. Oncogene 6:: 439-444.

2. Schuuring E, Verhoeven E, Mooi WJ, and Michalides RJAM (1992) Identification and cloning of two overexpressed genes, U21B31/PRAD1 and EMS1, within the amplified chromosome 11q13 region in human carcinomas. Oncogene 7: 355-361.

3. Theillet C, Adane J, Szepetowski P, Simon M-P, Jeanteur P, Birnbaum D, and Gaudray P (1990) BCL-1 participates in the 11q13 amplification found in breast cancer. Oncogene 5: 147-149.

4. Bartkova J, Lukas J, Muller H, Lutzhoft D, Strauss M, and Bartek J (1994) Cyclin D1 protein expression and function in human breast cancer. Int. J. Cancer 57:353-361.

5. Buckley MF, Sweeney KJE, Hamilton JA, Sini RL, Manning DL, Nicholson RI, deFazio A, Watts CKW, Musgrove EA, and Sutherland RL (1993) Expression and amplification of cyclin genes in human breast cancer. Oncogene 8: 2127-2133.

6. Wang TC, Cardiff RD, Zukerberg L, Lees E, Arnold A, and Schmidt EV (1994) Mammary hyperplasia and carcinoma in MMTV-cyclin D1 transgenic mice. Nature 369: 669-671.

7. Porter PL, Malone KE, Heagerty PJ, Alexander GM, Gatti LA, Firpo EJ, Daling JR, and Roberts JM. (1997) Expression of cell-cycle regulators p27kip1 and cyclin E, alone and in combination, correlate with survival in young breast cancer patients. Nature Medicine 3: 222-225.

8. Catzavelos C, Bhattacharya N, Ung YC, Wilson JA, Roncari L, Sandhu C, Shaw P, Yeger H, Morava-Protzner I, Kapusta L, Franssen E, Pritchard KI, and Slingerland JM. (1997) Decreased levels of the cell-cycle inhibitor p27kip1 protein: prognostic implications in primary breast cancer. Nature Medicine 3: 227-230.

9. Loda M, Cukor B, Tam SW, Lavin P Fiorentino M, Draetta GF, Jessup JM, and Pagano M. (1997) Increased proteasome-dependent degradation of the cyclin-dependent kinase inhibitor p27 in aggressive colorectal carcinomas. Nature Medicine 3: 231-234.

10. Fredersdorf S, Burns J, Milne AM, Packham G, Fallis L, Gillett CE, Royds JA, Peston D, Hall PA, Hanby AM, Barnes DM, Shousha S, O'Hare MJ, and Lu X (1997) High level expression of p27kip1 and cyclin D1in some human breast cancer cells: inverse correlation between the expression of p27kip1 and degree of malignancy in human breast and colorectal cancers. Proc. Natl. Acad. Sci. 94: 6380-6385.

11. Jiang M, Shao Z-M, Wu J, Lu J-S, Yu L-M, Yuan J-D, Han Q-X, Shen Z-Z, and Fontana JA (1997) p21/waf1/cip1 and mdm-2 expression in breast carcinoma patients as related to prognosis. Int. J. Cancer 74: 529-534.

12. Polyak K, Kato J-y, Solomon MJ, Sherr CJ, Massague J, Roberts JM, Koff A. (1994) p27kip1, a cyclin-cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. Genes and Development 8: 9-22.

13. Polyak K, Lee M-H, Erdjument-Bromage H, Koff A, Roberts JM, Tempst P, and Massague J. (1994) Cloning of p27kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. Cell 78: 59-66.

14. Toyoshima H and Hunter T. (1994) p27, a novel inhibitor of G1 cyclin-cdk protein kinase activity, is related to p21. Cell 78: 67-74.

15. Nourse J, Firpo E, Flanagan WM, Coats S, Polyak K, Lee M-H, Massague J, Crabtree GR, and Roberts JM. (1994) Interleukin-2-mediated elimination of the p27kip1 cyclin-dependent kinase inhibitor prevented by rapamycin. Nature 372: 570-573.

16. Sherr CJ and Roberts JR (1995) Inhibitors of mammalian cyclin-dependent kinases. Genes an Dev. 9: 1149-1163.

17. Zhu X, Ohtsubo M, Bohmer RM, Roberts JM, and Assoian RK. (1996) Adhesion-dependent cell cycle progression linked to the expression of cyclin D1, activation of cyclin E-cdk2, and phosphorylation of the retinoblastoma protein. J Cell Biol. 133: 391-403.

18. Fang F, Orend G, Watanabe N, Hunter T, and Ruoslahti E (1996) Dependence of cyclin E-cdk2 kinase activity on cell anchorage. Science 271: 499-502.

19. Han EK, Sgambato A, Jiang W, Zhang Y, Santella RM, Doki Y, Cacace AM, Schieren I, and Weinstein IB (1995) Stable overexpression of cyclin D1 in a human mammary epithelial cell line prolongs the S-phase and inhibits growth. Oncogene 10: 953-961.

20. Han EK, Begemann M, Sgambato A, Soh J-W, Doki Y, Xing W-Q, Liu W, and Weinstein IB (1996) Increased expression of cyclin D1 in a murine mammary epithelial cell line induces p27kip1, inhibits growth, and enhances apoptosis. Cell Growth and Diff. 7: 699-710.

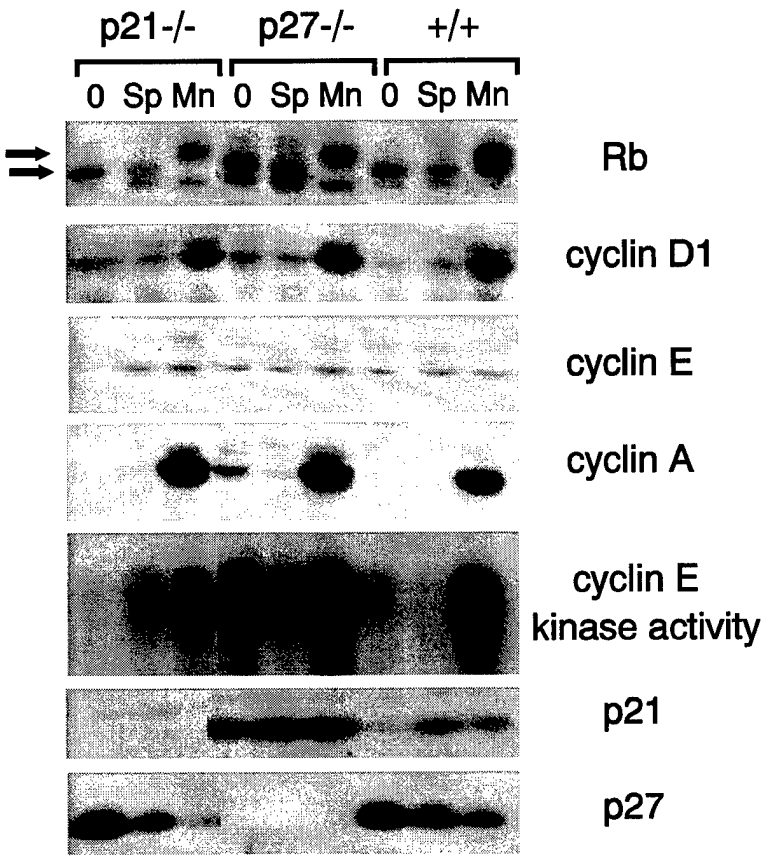
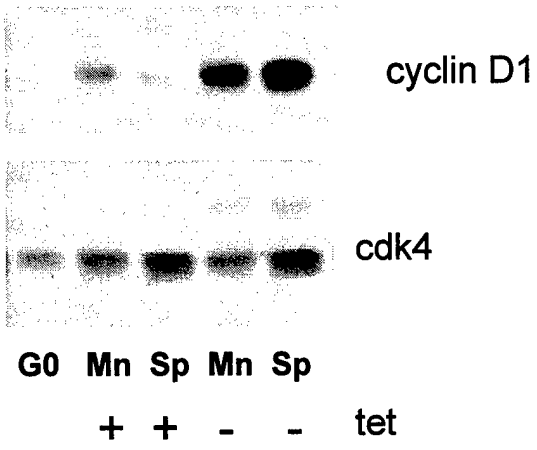


Fig. 1. The adhesion-dependent phenotype of p21-/- and p27-/- and wild-type (+/+) mouse embryo fibroblasts. *G0-synchronized (serum-starved) cells were exposed to mitogens (FCS/EGF) in suspension (Sp) or monolayer (Mn) for 18 h. Collected cells were extracted and the expression cyclin D1, cyclin E, cyclin A, p21 and p27 as well as the phosphorylation of Rb were determined by immunoblotting (lower and upper arrows show the positions of hypo and hyperphosphorylated Rb, respectively). Duplicate aliquots of the extract were used to assess cyclin E-cdk2 kinase activity in vitro as described in Zhu et al. Note that the activation of cyclin E kinase in the absence of cyclin D1 (e.g. in G0-synchronized p27-/- cells and in suspension cultures of p21-/- cells) failed to result in Rb phosphorylation as determined by gel-shift.*



Tet-regulated expression of cyclin D1. *MEFs were transfected with vectors conferring tet-regulated (tet-off) cyclin D1 expression. Stable transfectants were isolated by drug selection. Clone 14 is shown. Serum starved cells (G0) were stimulated with 10% FCS in monolayer (Mn) and suspension (Sp) for 16 hr in the presence and absence of 2 ug/ml tetracycline. Cell extracts were immunoblotted with anti-cyclin D1 and cdk4 (loading control).*

FIGURE 2

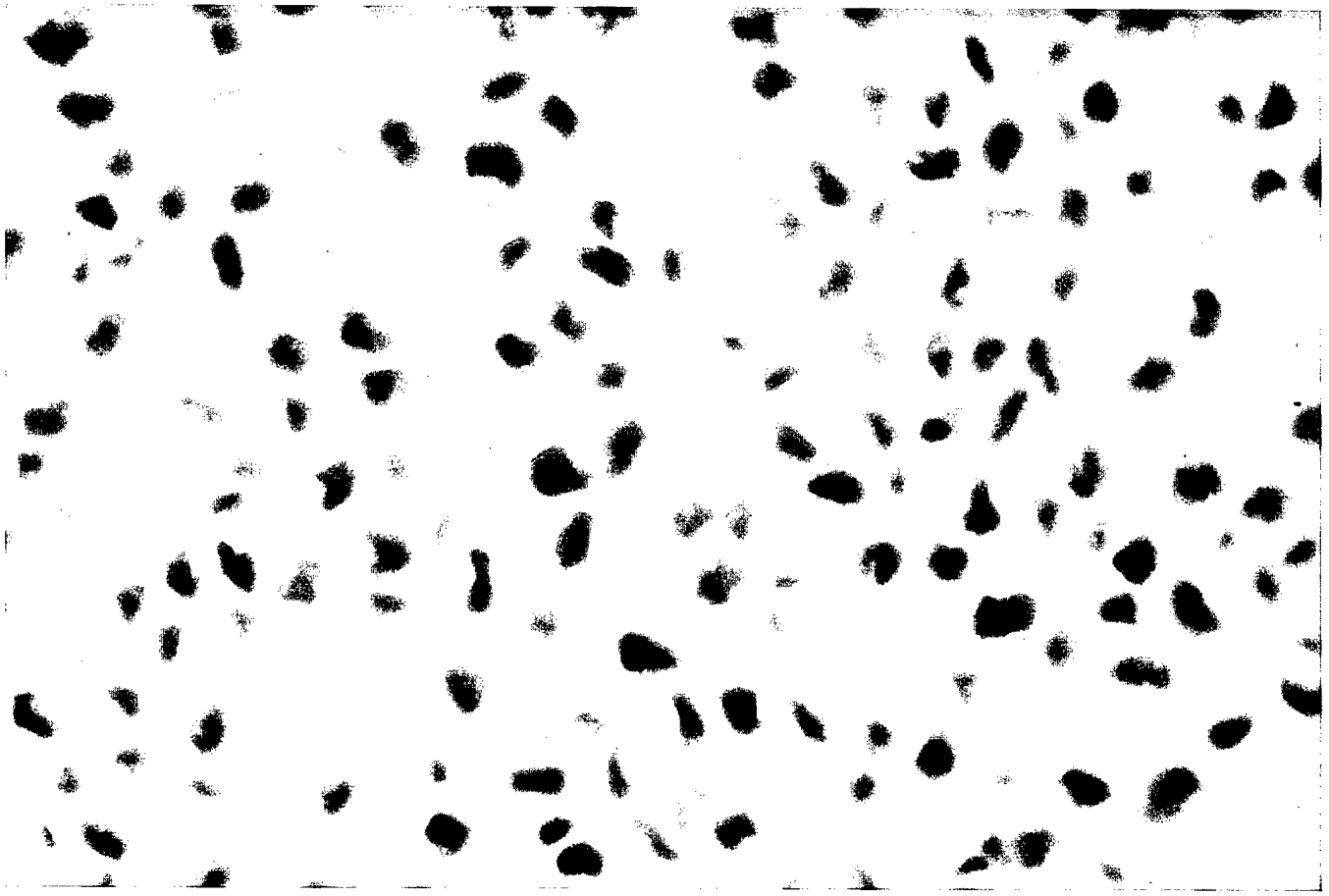


Fig 3. Anti-p27 IHC staining (+) control. All high-p27 expression control cells cells exhibit strong nuclear staining under optimized conditions: Paraffin sections were incubated overnight at room temperature with p27 antibody (1:10,000) after a 20 min steam heat antigen retrieval (AR) step in 0.01 M citrate buffer, pH 9. Tris and citrate buffers, each at three pHs, were systematically tested in the optimization protocol, as were duration of AR, antibody dilution and incubation interval (600 X original magnification).

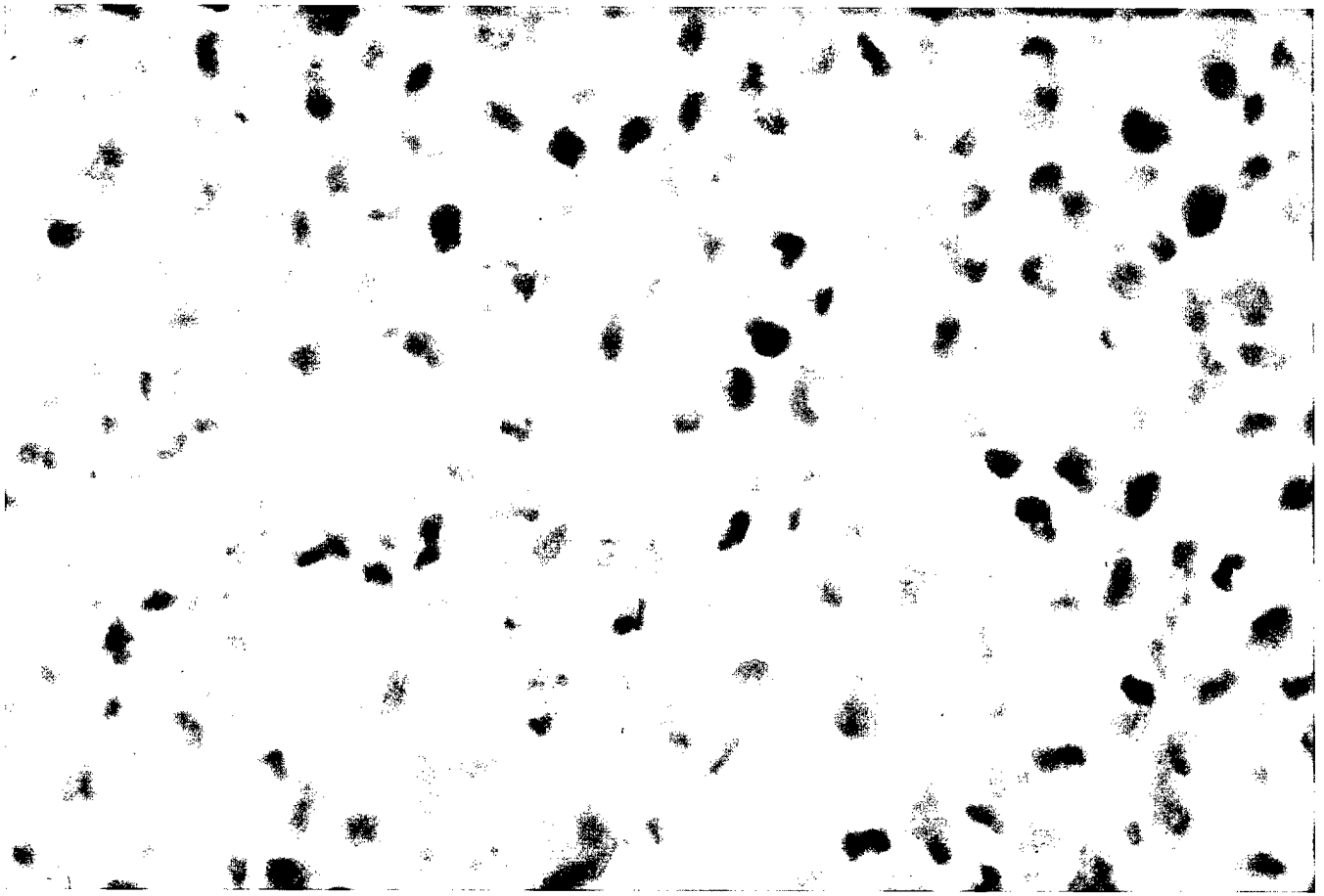


Fig 4. Anti-p27 IHC staining (+) control. Approximately 95% of these low p27 expression control cells exhibit 0 - 2 + nuclear staining under the optimized conditions. (600 X original magnification).

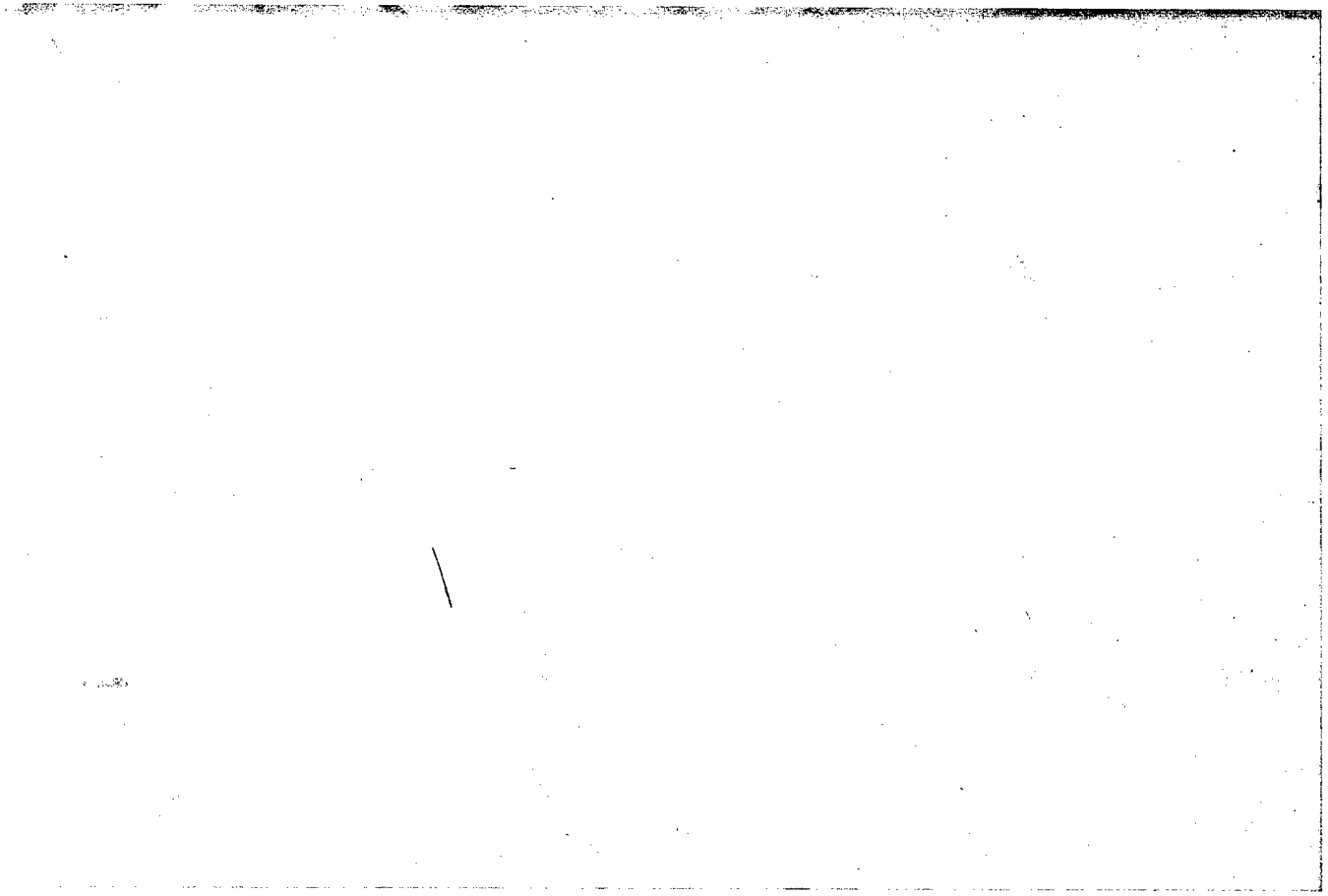


Fig 5. Anti-p27 IHC staining negative control. Example of the lack of nuclear staining that results when the primary antibody is omitted when evaluating sections of formalin fixed, paraffin embedded control cells, both high and low expressors (600 X original magnification).

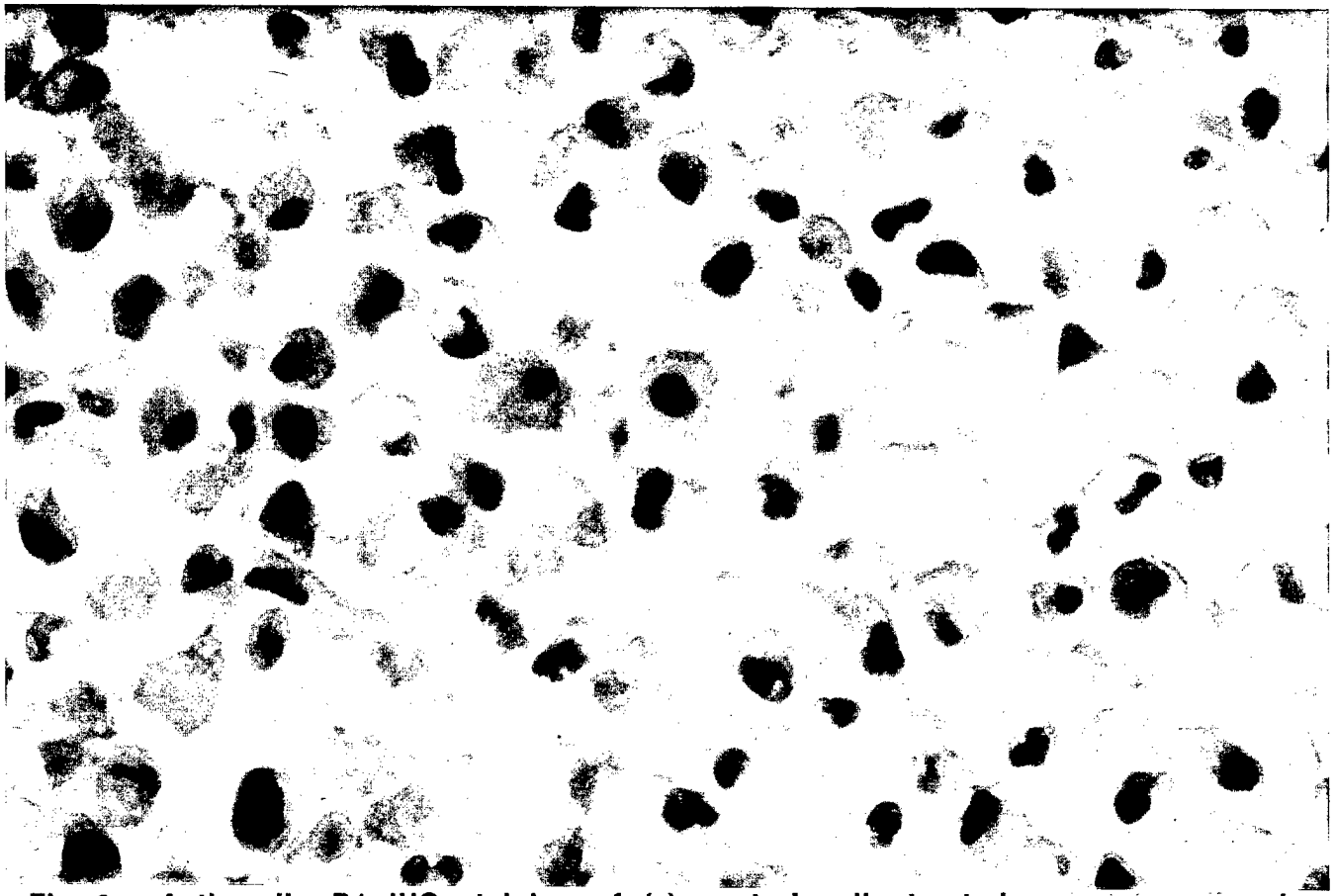


Fig 6 Anti-cyclin D1 IHC staining of (+) control cells treated to induce cyclin D1 expression. All cells cells exhibit strong nuclear staining under optimized conditions: Paraffin sections were incubated overnight at room temperature with cyclin D1 antibody (Dako: 1:250) after a 20 min steam heat antigen retrieval (AR) step in a Tris HCl buffer, pH 6. Tris and citrate buffers, each at three pHs, were systematically tested in the optimization protocol, as were duration of AR, antibody dilution and incubation interval (600 X original magnification).

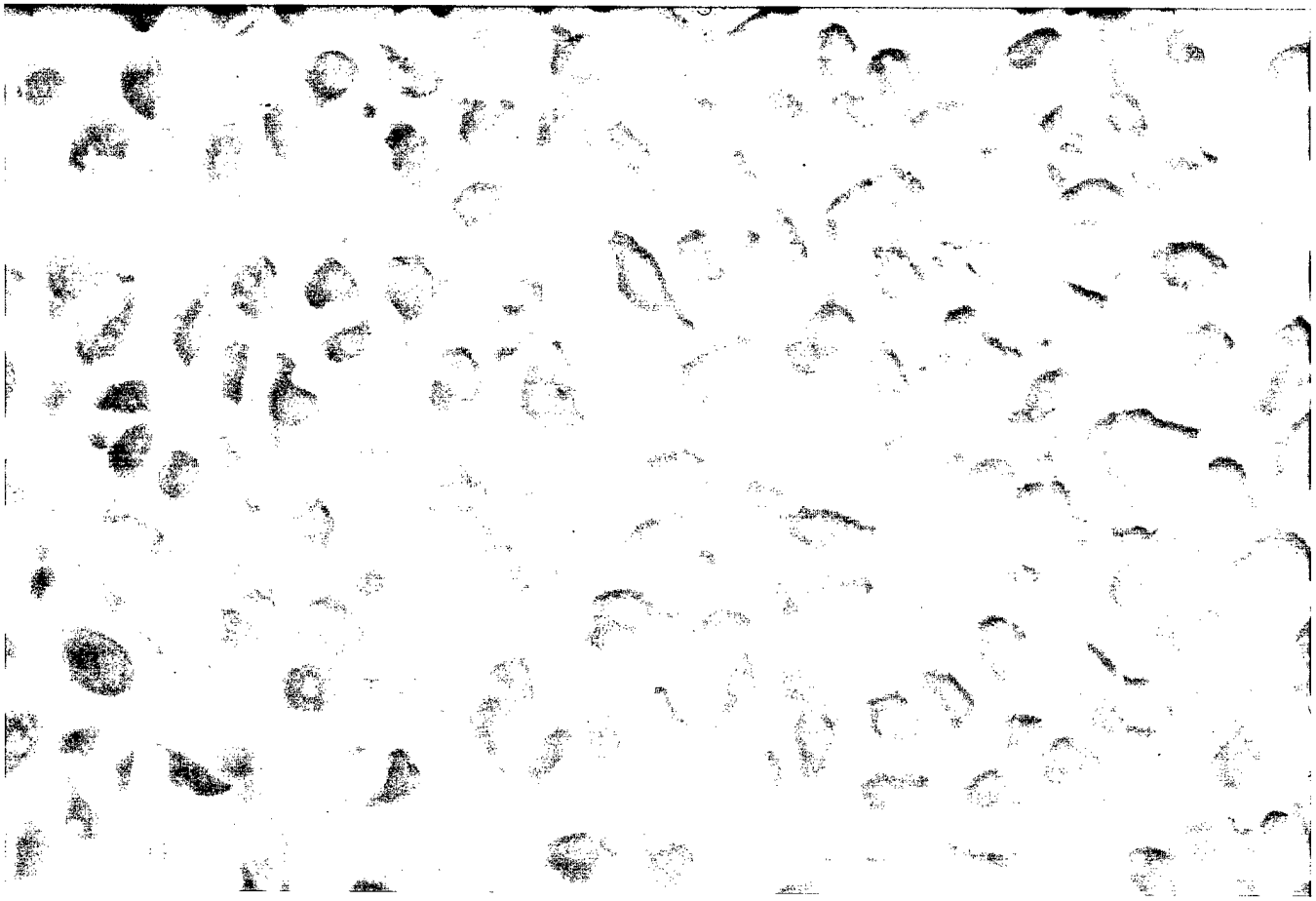


Fig 7 Anti-cyclin D1 in tetracyclin treated (-) control cells. No nuclear staining is seen under the optimized IHC staining conditions. (600 X original magnification).

Fig 8 Anti-cyclin D1 IHC staining negative control. There is no nuclear staining when the primary antibody is omitted from sections of formalin fixed, paraffin embedded control cells that have been treated with tetracycline to induce cyclin D1 expression (400 X original magnification).